(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 April 2000 (27.04.2000)

PCT

(10) International Publication Number
WO 00/23114 A3

(51) International Patent Classification[7]: A61K 47/48, 38/19

(21) International Application Number: PCT/US99/24201

(22) International Filing Date: 15 October 1999 (15.10.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/104,572 16 October 1998 (16.10.1998) US
60/120,161 16 February 1999 (16.02.1999) US

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(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
28 September 2000

(48) Date of publication of this corrected version:
5 July 2001

(15) Information about Correction:
see PCT Gazette No. 27/2001 of 5 July 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/23114 A3

(54) Title: POLYMER CONJUGATES OF INTERFERON BETA- 1A AND THEIR USES

(57) Abstract: An interferon beta polypeptide comprising interferon-beta 1a coupled to a polymer containing a polyalkylene glycol moiety wherein the interferon-beta-1a and the polyalkylene glycol moiety are arranged such that the interferon-beta-1a has an enhanced activity relative to another therapeutic form of interferon beta (interferon-beta-1b) and exhibits no decrease in activity as compared to non-conjugated interferon-beta-1a. The conjugates of the invention are usefully employed in therapeutic as well as non-therapeutic, e.g., diagnostic, applications.

hereinafter, "interferon-beta-1b". Interferon beta-1a is produced in mammalian cells using the natural human gene sequence and is glycosylated, whereas interferon beta-1b is produced in *E. coli* bacteria using a modified human gene sequence that contains a genetically engineered cysteine-to-serine substitution at amino acid position 17 and is non-glycosylated.

Previously, several of us have directly compared the relative *in vitro* potencies of interferon-beta-1a and interferon beta 1b in functional assays and showed that the specific activity of interferon-beta-1a is approximately 10-fold greater than the specific activity of interferon-beta-1b (Runkel et al., 1998, Pharm. Res. 15: 641-649). From studies designed to identify the structural basis for these activity differences, we identified glycosylation as the only one of the known structural differences between the products that affected the specific activity. The effect of the carbohydrate was largely manifested through its stabilizing role on structure. The stabilizing effect of the carbohydrate was evident in thermal denaturation experiments and SEC analysis. Lack of glycosylation was also correlated with an increase in aggregation and an increased sensitivity to thermal denaturation. Enzymatic removal of the carbohydrate from interferon-beta-1a with PNGase F caused extensive precipitation of the deglycosylated product.

These studies indicate that, despite the conservation in sequence between interferon-beta-1a and interferon-beta-1b, they are distinct biochemical entities and therefore much of what is known about interferon-beta-1b cannot be applied to interferon-beta-1a, and vice versa.

SUMMARY OF THE INVENTION

We have exploited the advantages of glycosylated interferon-beta relative to non-glycosylated forms. In particular, we have developed an interferon-beta-1a composition with increased activity relative to interferon-beta-1b and that also has the salutory properties of pegylated proteins in general with no effective loss in activity as compared to interferon-beta-1a forms that are not conjugated. Thus, if modifications are made in such a way that the products (polymer-interferon-beta 1a conjugates) retain all or most of their biological activities, the following properties may result: altered pharmacokinetics and pharmacodynamics leading to increased half-life and alterations in tissue distribution (e.g,

In another aspect, the present invention relates to a physiologically active interferon-beta-1a composition comprising physiologically active interferon-beta-1a coupled with a polymer comprising a polyalkylene glycol moiety wherein the interferon-beta-1a and polyalkylene glycol moiety are arranged such that the physiologically active interferon-beta-1a in the composition has an enhanced activity relative to interferon-beta-1b alone (i.e., in an unconjugated form devoid of the polymer coupled thereto).

Another embodiment of the invention is a conjugated interferon-beta-1a protein whose interferon-beta-1a moiety has been mutated to provide for muteins with selectively enhanced antiviral and/or antiproliferative activity relative to non-mutated forms of interferon-beta-1a.

The invention relates to a further aspect to a stable, aqueously soluble, conjugated interferon-beta-1a complex comprising a physiologically active interferon-beta-1a covalently coupled to a physiologically compatible polyethylene glycol moiety. In such complex, the interferon-beta-1a may be covalently coupled to the physiologically compatible polyethylene glycol moiety by a labile covalent bond at a free amino acid group of the interferon-beta-1a, wherein the labile covalent bond is severed in vivo by biochemical hydrolysis and/or proteolysis.

In another aspect, the present invention relates to a dosage form comprising a pharmaceutically acceptable carrier and a stable, aqueously soluble, interferon-beta 1a complex comprising interferon-beta coupled to a physiologically compatible polyethylene glycol.

In another aspect, covalently coupled interferon-beta-1a compositions such as those described above may utilize interferon-beta-1a intended for diagnostic or in vitro applications, wherein the interferon-beta-1a is for example a diagnostic reagent for immunoassay or other diagnostic or non-in vivo applications. In such non-therapeutic applications, the complexes of the invention are highly usefully employed as stabilized compositions which may for example be formulated in compatible solvents or other solution-based formulations to provide stable compositional forms which are of enhanced resistance to degradation.

fusion protein comprising the extracellular domain of the type I interferon receptor chain, IFNAR2/Ig (IFNAR2 ectodomain fused to the human IgG1 constant domain.

The binding affinities of the alanine substituted IFN mutants (A1 - E) for the IFNAR2 receptor chain were determined as described in Example 1 (subsection D). The histogram presents their binding affinities in this assay relative to wild type his-IFN-beta (% w.t.). The % w. t. values were calculated as the (affinity of wild type his-IFN-beta)/ affinity of mutant IFN-beta x 100. The % w. t. (O) for individual experiments (n = 3) and an average % w.t. (x) for the experimental set are shown. Mutants A2, AB1, AB2, and E did not bind IFNAR2/Fc at concentrations 500-fold higher than the w.t. his-IFN-beta EC 50 (*).

Figure 2. **Binding of alanine substituted interferon-beta-1a mutants to the type I interferon cell surface receptor complexes ("IFNAR1/2 complex") expressed on Daudi Burkitt's lymphoma cells.** The receptor binding properties of the alanine substitution mutants (A1 - E) were determined using a FACS based, cell surface receptor binding assay as described in Example 1(subsection D). The histogram presents their receptor binding affinities in this assay relative to wild type his-IFN-beta (% w.t.). The % w. t. for each mutant was calculated as the (affinity of wild type his-IFN-beta)/ affinity of mutant IFN-beta x 100. The % w.t. values (O) for individual experiments and an average of the % w.t. values for the experimental set (x) are shown.

Figure 3. **Antiviral activities of alanine substituted interferon-beta-1a mutants**

The antiviral activities of the alanine substitution mutants (A1- E) were determined on human A549 cells challenged with EMC virus as described in Example 1 (subsection E). The histogram presents their activities in this assay relative to wild type his-IFN-beta (% w.t.). The % w. t. was calculated as the (concentration of w.t. his-IFN-beta [50% cpe]/concentration of mutant IFN-beta [50% cpe] x100. The % w.t (o) for multiple assays and the average of the experimental data set (x) are shown.

Figure 4. **Antiproliferative activities of alanine substituted interferon-beta-1a mutants** The antiproliferation activity of the alanine substitution mutants (A1 - E) were

nm, and the absorbance which is reflective of cell viability is shown on the Y axis. The standard deviations are shown as error bars. The concentration of interferon-beta-1a or PEGylated interferon beta-1a which offered 50% viral killing (the "50% cytopathic effect") (50% maximum OD450) was about 11 pg/ml and the 50% cytopathic effect for PEGylated interferon-beta-1a was about 11pg/ml.

Figure 8. **Assessing stabilization of conjugates using thermal denaturation**

PEGylated interferon-beta-1a and untreated interferon-beta-1a control in 20mM HEPES pH 7.5, 20mM NaCl were heated at a fixed rates of 1 degree/min. Denaturation was followed by monitoring absorbance changes at 280nm. (a) unmodified interferon-beta-1a (b) PEGylated interferon-beta-1a.

Figure 9. **Measurements of interferon-beta antiviral activity in the plasma of mice treated with interferon-beta-1a or PEGylated interferon-beta-1a.**

Mice are injected iv with either 50,000 Units of interferon-beta-1a or 50,000 Units of pegylated-interferon-beta-1a (containing the 20K PEG). Blood from these mice is obtained via retro-orbital bleeds at various times after interferon injection as indicated on the X axis. There are at least 3 mice bled at each time point, and plasma is prepared and frozen until the time interferon-beta activity is evaluated in antiviral assays using human lung carcinoma (A549) cells challenged with encephalomyocarditis virus. Viable cells were stained with a solution of MTT, the plates were read at 450 nm, to determine the absorbance which is reflective of cell viability and interferon-beta activity. Standard curves were generated for each plate using interferon-beta-1a and used to determine the amount of interferon-beta activity in each sample. Data from the individual animals are shown.

Figure 10. **Full DNA sequence of histidine-tagged interferon beta gene and its protein product.** The full DNA (SEQ ID NO: 1) and protein (SEQ ID NO: 2) sequences of the histidine-tagged IFN-beta-1a are shown. The cleaved VCAM-1 signal sequence leaves 3 amino terminal residues (SerGlyGly) upstream of the histidine tag (His_6, positions

lymphoblastoid interferons LyIFN- alpha -2 and LyIFN- alpha -3); **U.S. Patent 4,970,161** (Nov. 13, 1990, DNA sequence coding for human interferon- gamma); **U.S. Patent 4,738,931** (Apr. 19, 1988, DNA containing a human interferon beta gene); **U.S. Patent 4,695,543** (Sep. 22, 1987, human alpha-interferon Gx-1 gene and U.S. Patent **4,456,748** (Jun. 26, 1984, DNA encoding sub-sequences of different, naturally, occurring leukocyte interferons).

Mutants of interferon-beta-1a may be used in accordance with this invention. Mutations are developed using conventional methods of directed mutagenesis, known to those of ordinary skill in the art. Moreover, the invention provides for functionally equivalent interferon-beta-1a polynucleotides that encode for functionally equivalent interferon-beta-1a polypeptides.

A first polynucleotide encoding interferon-beta-1a is "functionally equivalent" compared with a second polynucleotide encoding interferon-beta-1a if it satisfies at least one of the following conditions:

(a): the "functional equivalent" is a first polynucleotide that hybridizes to the second polynucleotide under standard hybridization conditions and/or is degenerate to the first polynucleotide sequence. Most preferably, it encodes a mutant interferon having the activity of an interferon-beta-1a;

(b) the "functional equivalent" is a first polynucleotide that codes on expression for an amino acid sequence encoded by the second polynucleotide.

In summary, the term "interferon" includes, but is not limited to, the agents listed above as well as their functional equivalents. As used herein, the term "functional equivalent" therefore refers to an interferon-beta-1a protein or a polynucleotide encoding the interferon-beta-1a protein that has the same or an improved beneficial effect on the mammalian recipient as the interferon of which it is deemed a functional equivalent. As will be appreciated by one of ordinary skill in the art, a functionally equivalent protein can be produced by recombinant techniques, e.g., by expressing a "functionally equivalent DNA". Accordingly, the instant invention embraces interferon-beta-1a proteins encoded by naturally-occurring DNAs, as well as by non-naturally-occurring DNAs which encode the same protein as encoded by the naturally-occurring DNA. Due to the degeneracy of the

A "therapeutic composition" as used herein is defined as comprising the proteins of the invention and other physiologically compatible ingredients. The therapeutic composition may contain excipients such as water, minerals and carriers such as protein.

An "effective amount" of an agent of the invention is that amount which produces a result or exerts an influence on the particular condition being treated.

"amino acid"- a monomeric unit of a peptide, polypeptide, or protein. There are twenty amino acids found in naturally occurring peptides, polypeptides and proteins, all of which are L-isomers. The term also includes analogs of the amino acids and D-isomers of the protein amino acids and their analogs.

A "derivatized" amino acid is a natural or nonnatural amino acid in which the normally occurring side chain or end group is modified by chemical reaction. Such modifications include, for example, gamma-carboxylation, beta-carboxylation, sulfation, sulfonation, phosphorylation, amidization, esterification, N-acetylation, carbobenzylation, tosylation, and other modifications known in the art. A "derivatized polypeptide" is a polypeptide containing one or more derivatized amino acids.

"protein"- any polymer consisting essentially of any of the 20 amino acids. Although "polypeptide" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and is varied. The term "protein" as used herein refers to peptides, proteins and polypeptides, unless otherwise noted.

"mutant" - any change in the genetic material of an organism, in particular any change (i.e., deletion, substitution, addition, or alteration) in a wild-type polynucleotide sequence or any change in a wild-type protein. The term "mutein" is used interchangeably with "mutant".

"wild-type" - the naturally-occurring polynucleotide sequence of an exon of a protein, or a portion thereof, or protein sequence, or portion thereof, respectively, as it normally exists *in vivo*.

"standard hybridization conditions"- salt and temperature conditions substantially equivalent to 0.5 X SSC to about 5 X SSC and 65 ° C for both hybridization and wash. The term "standard hybridization conditions" as used herein is therefore an operational

expression vector, or a portion thereof); or (ii) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By "isolated" it is further meant a polynucleotide sequence that is: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) chemically synthesized; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and gel separation.

Thus, "substantially pure nucleic acid" is a nucleic acid which is not immediately contiguous with one or both of the coding sequences with which it is normally contiguous in the naturally occurring genome of the organism from which the nucleic acid is derived. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding additional sequences.

"Isolated" (used interchangeably with "substantially pure")- when applied to polypeptides means a polypeptide or a portion thereof which, by virtue of its origin or manipulation: (i) is present in a host cell as the expression product of a portion of an expression vector; or (ii) is linked to a protein or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By "isolated" it is further meant a protein that is : (i) chemically synthesized; or (ii) expressed in a host cell and purified away from associated proteins. The term generally means a polypeptide that has been separated from other proteins and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also separated from substances such as antibodies or gel matrices (polyacrylamide) which are used to purify it.

"heterologous promoter"- as used herein is a promoter which is not naturally associated with a gene or a purified nucleic acid.

"Homologous"- as used herein is synonymous with the term "identity" and refers to the sequence similarity between two polypeptides, molecules or between two nucleic acids. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit (for instance, if a position in each of the two DNA molecules is occupied by adenine, or a position in each of two polypeptides is occupied by a lysine), then the respective molecules are homologous at that position. The percentage homology between two sequences is a function of the number of matching or homologous positions

Cold Spring Harbor Laboratory Press, 1989; **DNA Cloning**, Volumes I and II (D.N. Glover, ed), 1985; **Oligonucleotide Synthesis**, (M.J. Gait, ed.), 1984; U.S. Patent No. 4,683,195 (Mullis et al.,); **Nucleic Acid Hybridization** (B.D. Hames and S.J. Higgins, eds.), 1984; Transcription and Translation (B.D. Hames and S.J. Higgins, eds.), 1984; **Culture of Animal Cells** (R.I. Freshney, ed). Alan R. Liss, Inc., 1987; **Immobilized Cells and Enzymes,** IRL Press, 1986; **A Practical Guide to Molecular Cloning** (B. Perbal), 1984; **Methods in Enzymology,** Volumes 154 and 155 (Wu et al., eds), Academic Press, New York; **Gene Transfer Vectors for Mammalian Cells** (J.H. Miller and M.P. Calos, eds.), 1987, Cold Spring Harbor Laboratory; **Immunochemical Methods in Cell and Molecular Biology** (Mayer and Walker, eds.), Academic Press, London, 1987; **Handbook of Experiment Immunology,** Volumes I-IV (D.M. Weir and C.C. Blackwell, eds.), 1986; **Manipulating the Mouse Embryo,** Cold Spring Harbor Laboratory Press, 1986.

THE INTERFERON-BETA

Interferon-beta-1a is useful as an agent for the treatment, remission or attenuation of a disease state, physiological condition, symptoms, or etiological factors, or for the evaluation or diagnosis thereof. The term also refers to interferon-beta-1a that is itself part of a fusion protein such as an immunoglobulin-interferon-beta-1a fusion protein, as described in co-pending applications Serial Numbers 60/104,572 and 60/120,161. Preparation of fusion proteins generally are well within the knowledge of persons having ordinary skill in the art.

We found unique site(s) for polymer attachment that would not destroy function of the interferon-beta-1a. In addition, we also used site-directed mutagenesis methods to independently investigate site(s) for polymer attachment (See Example 1). Briefly, we undertook a mutational analysis of human interferon-beta-1a with the aim of mapping residues required for activity and receptor binding. The availability of the 3-D crystal structure of human interferon-beta-1a (see above and Example 1) allows us to identify, for alanine (or serine) substitutions, the solvent-exposed residues available for interferon beta receptor interactions, and to retain amino acids involved in intramolecular bonds. A panel

applications. Additionally, it will be recognized that the conjugating polymer may utilize any other groups, moieties, or other conjugated species, as appropriate to the end use application. By way of example, it may be useful in some applications to covalently bond to the polymer a functional moiety imparting UV-degradation resistance, or antioxidation, or other properties or characteristics to the polymer. As a further example, it may be advantageous in some applications to functionalize the polymer to render it reactive or cross-linkable in character, to enhance various properties or characterisics of the overall conjugated material. Accordingly, the polymer may contain any functionality, repeating groups, linkages, or other constitutent structures which do not preclude the efficacy of the conjugated interferon-beta 1a composition for its intended purpose. Other objectives and advantages of the present invention will be more fully apparent from the ensuing disclosure and appended claims.

Illustrative polymers that may usefully be employed to achieve these desirable characteristics are described herein below in exemplary reaction schemes. In covalently bonded peptide applications, the polymer may be functionalized and then coupled to free amino acid(s) of the peptide(s) to form labile bonds.

The interferon-beta-1a is conjugated most preferably via a terminal reactive group on the polymer although conjugations can also be branched from the non-terminal reactive groups. The polymer with the reactive group(s) is designated herein as "activated polymer". The reactive group selectively reacts with free amino or other reactive groups on the protein. The activated polymer(s) are reacted so that attachment may occur at any available interferon-beta-1a amino group such as the alpha amino groups or the epsilon-amino groups of lysines. Free carboxylic groups, suitably activated carbonyl groups, hydroxyl, guanidyl, oxidized carbohydrate moieties and mercapto groups of the interferon-beta-1a (if available) can also be used as attachment sites.

Although the polymer may be attached anywhere on the interferon-beta 1a molecule, the most preferred site for polymer coupling is the N-terminus of the interferon-beta-1a. Secondary site(s) are at or near the C-terminus and through sugar moieties. Thus, the invention contemplates as its most preferred embodiments: (i) N-terminally coupled polymer conjugates of interferon-beta-1a; (ii) C-terminally coupled polymer conjugates of

reacted with interferon-beta-1a in the presence of sodium cyanoborohydride. This results, after purification of the PEG-interferon-beta-1a and analysis with SDS-PAGE, MALDI mass spectrometry and peptide sequencing/mapping, resulted in an interferon-beta-1a whose N-terminus is specifically targeted by the PEG moiety.

The crystal structure of interferon-beta-1a us such that the N- and C-termini are located close to each other (see Karpusas et al., 1997, Proc. Natl. Acad. Sci. 94: 11813-11818). Thus, modifications of the C- terminal end of interferon-beta-1a should also have minimal effect on activity. While there is no simple chemical strategy for targeting a polyalkylene glycol polymer such as PEG to the C-terminus, it would be straightforward to genetically engineer a site that can be used to target the polymer moiety. For example, incorporation of a Cys at a site that is at or near the C-terminus would allow specific modification using a maleimide, vinylsulfone or haloacetate- activated polyalkylene glycol (e.g., PEG). These derivatives can be used specifically for modification of the engineered cysteines due to the high selectively of these reagents for Cys. Other strategies such as incorporation of a histidine tag which can be targeted (Fancy et al., (1996) Chem. & Biol. 3: 551) or an additional glycosylation site, represent other alternatives for modifying the C-terminus of interferon-beta-1a.

The glycan on the interferon-beta-1a is also in a position that would allow further modification without altering activity. Methods for targeting sugars as sites for chemical modification are also well known and therefore it is likely that a polyalkylene glycol polymer can be added directly and specifically to sugars on interferon-beta-1a that have been activated through oxidation. For example, a polyethyleneglycol-hydrazide can be generated which forms relatively stable hydrazone linkages by condensation with aldehydes and ketones. This property has been used for modification of proteins through oxidized oligosaccharide linkages. See Andresz, H. et al., (1978), Makromol. Chem. 179: 301. In particular, treatment of PEG-carboxymethyl hydrazide with nitrite produces PEG-carboxymethyl azide which is an electrophilically active group reactive toward amino groups. This reaction can be used to prepare polyalkylene glycol-modified proteins as well. See, U.S. Patents 4,101,380 and 4,179,337.

illustrative and that all polymer materials having the qualities described herein are contemplated.

The polymer need not have any particular molecular weight, but it is preferred that the molecular weight be between about 300 and 100,000, more preferably between 10,000 and 40,000. In particular, sizes of 20,000 or more are best at preventing protein loss due to filtration in the kidneys.

Polyalkylene glycol derivatization has a number of advantageous properties in the formulation of polymer-interferon-beta 1a conjugates in the practice of the present invention, as associated with the following properties of polyalkylene glycol derivatives: improvement of aqueous solubility, while at the same time eliciting no antigenic or immunogenic response; high degrees of biocompatibility; absence of in vivo biodegradation of the polyalkylene glycol derivatives; and ease of excretion by living organisms.

Moreover, in another aspect of the invention, one can utilize interferon-beta 1a covalently bonded to the polymer component in which the nature of the conjugation involves cleavable covalent chemical bonds. This allows for control in terms of the time course over which the polymer may be cleaved from the interferon-beta 1a. This covalent bond between the interferon-beta-1a drug and the polymer may be cleaved by chemical or enzymatic reaction. The polymer-interferon-beta-1a product retains an acceptable amount of activity. Concurrently, portions of polyethylene glycol are present in the conjugating polymer to endow the polymer-interferon-beta-1a conjugate with high aqueous solubility and prolonged blood circulation capability. As a result of these improved characteristics the invention contemplates parenteral, nasal, and oral delivery of both the active polymer-interferon-beta-1a species and, following hydrolytic cleavage, bioavailability of the interferon-beta-1a per se, in in vivo applications.

It is to be understood that the reaction schemes described herein are provided for the purposes of illustration only and are not to be limiting with respect to the reactions and structures which may be utilized in the modification of the interferon-beta-1a, e.g., to achieve solubility, stabilization, and cell membrane affinity for parenteral and oral administration. The reaction of the polymer with the interferon-beta 1a to obtain the most

need of such treatment, comprising administering to such animal an effective amount of a polymer conjugate of the present invention which is therapeutically effective for said condition or disease state. Subjects to be treated by the polymer conjugates of the present invention include mammalian subjects and most preferably human subjects. Depending on the specific condition or disease state to be combated, animal subjects may be administered polymer conjugates of the invention at any suitable therapeutically effective and safe dosage, as may readily be determined within the skill of the art, and without undue experimentation. Because of the species barriers of Type I interferons, it may be necessary to generate interferon-polymer conjugates as described herein with interferons from the appropriate species.

The anti-cell proliferative activity of interferon-beta-1a is well known. In particular, certain of the interferon-beta-1a polymer conjugates described herein are useful for treating tumors and cancers such as osteogenic sarcoma, lymphoma, acute lymphocytic leukemia, breast carcinoma, melanoma and nasopharyngeal carcinoma, as well as autoimmune conditions such as fibrosis, lupus and multiple sclerosis. It is further expected that the anti-viral activity exhibited by the conjugated proteins, in particular certain of the interferon-beta-1a mutein conjugates described herein, may be used in the treatment of viral diseases, such as ECM infection, influenza, and other respiratory tract infections, rabies, and hepatitis. It is also expected that immunomodulatory activities of interferon-beta-1a exhibited by the conjugated proteins described herein, may be used in the treatment of autoimmune and inflammatory diseases, such as fibrosis, multiple sclerosis. The ability of interferons to inhibit formation of new blood vessels (i.e., inhibit angiogenesis and neovascularization) enables conjugates of the invention to be used to treat angiogenic diseases such as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis and Osler-Webber Syndrome.

Moreover, the antiendothelial activity of interferon has been known for some time and one potential mechanism of interferon action may be to interfere with endothelial cell activity by inhibiting the production or efficacy of angiogenic factors produced by tumor cells. Some vascular tumors, such as hemangiomas, are particularly sensitive to treatment

a gaseous dispersion of the powder which is inspired by the patient from a breathing circuit comprising a suitable nebulizer device.

The formulations comprising the polymer conjugates of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the active ingredient(s) into association with a carrier which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the active ingredient(s) into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the active ingredient as a powder or granules; or a suspension in an aqueous liquor or a non-aqueous liquid, such as a syrup, an elixir, an emulsion, or a draught.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, with the active compound being in a free-flowing form such as a powder or granules which optionally is mixed with a binder, disintegrant, lubricant, inert diluent, surface active agent, or discharging agent. Molded tablets comprised of a mixture of the powdered polymer conjugates with a suitable carrier may be made by molding in a suitable machine.

A syrup may be made by adding the active compound to a concentrated aqueous solution of a sugar, for example sucrose, to which may also be added any accessory ingredient(s). Such accessory ingredient(s) may include flavorings, suitable preservative, agents to retard crystallization of the sugar, and agents to increase the solubility of any other ingredient, such as a polyhydroxy alcohol, for example glycerol or sorbitol.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active conjugate, which preferably is isotonic with the blood of the recipient (e.g., physiological saline solution). Such formulations may include

ordinary skill in the art could use other neopterin assays or could alter the number and kind of primate used. These modifications and variations to the Examples are to be regarded as being within the spirit and scope of the invention.

EXAMPLE 1: **Structure/activity studies of human interferon-beta-1a using alanine/serine substitution mutations: Analysis of receptor binding sites and functional domains**

A. Overview

An extensive mutational analysis of human interferon-beta-1a (IFN-beta-1a) was undertaken with the aims of mapping residues required for activity and receptor binding. The availability of the 3-D crystal structure of human IFN-beta (Karpusas, M. et al. 1997, Proc. Natl. Acad. Sci. 94: 11813-11818) allowed us to identify for alanine (or serine) substitutions the solvent-exposed residues available for receptor interactions, and to retain amino acids involved in intramolecular bonds. A panel of 15 alanine substitution mutations were designed that replaced between 2 and 8 residues along distinct regions of each of the helices (A, B, C, D, E) and loops (AB, CD, DE). An amino-terminal histidine tag comprising six histidine residues was included for affinity purification, as well as an enterokinase cleavage site for removal of the amino-terminal extension. The resulting interferons are referred to as "his tagged-interferon(IFN)-beta" or "His-interferon-beta" or "His_6-interferon-beta" and the like.

Various mutant his tagged-IFN-beta expression plasmids were constructed using a wild type IFN-beta gene construct as a template for mutagenesis. The mutagenesis strategy involved first introducing unique restriction enzyme cleavage sites throughout the wild type his tagged-IFN beta gene, then replacing distinct DNA sequences between the chosen restriction sites with synthetic oligonucleotide duplexes, which encoded the alanine (or serine) substitution mutations. Finally, the mutant IFN genes were subcloned into a plasmid which directed mammalian cell expression in a human 293 kidney cell line.

Functional consequences of these mutations were assessed in antiviral and antiproliferation assays. A non-radioactive IFN binding assay was developed to analyze these mutants in their binding to the surface receptor ("IFNAR1/2 complex") of human

l/histidine tag cassette moiety were KID-369 (5' PCR primer 5'-AGCTTCCGGGGGCCATCATCATCATCATCATAGCT-3': SEQ ID NO: 5) and KID-421 (3' PCR primer 5'-CCGGAGCTATGATGATGATGATGATGG CCCCCGGA-3': SEQ ID NO:6) incorporating flanking restriction enzyme cleavage sites (NotI and BspEI) that allowed excision of the fragment B DNA.

To create a plasmid vector that carried the VCAM-1 signal sequence, his tag and interferon-beta gene we performed a three-way ligation using gel purified DNA fragments from plasmid vector pMJB107 (NotI and XhoI cleaved), PCR fragment A (BspEI and XhoI cleaved) and fragment B (NotI and BspEI cleaved). The ligated plasmid was used to transform either JA221 or XL1-Blue E. coli cells and ampicillin resistant colonies were picked and tested for inserts by restriction map analysis. Maxiprep DNA was made and the sequence of the insert was verified by DNA sequencing. The resulting construct was called pCMG260.

2. Creation of alanine substitution mutants of human interferon-beta in pCMG260

The plasmid pCMG260 was used as a template for multiple rounds of mutagenesis (U.S.E. Site Directed Mutagenesis Kit (Boehringer-Mannheim), which introduced unique restriction cleavage sites into positions along the IFN-beta protein coding sequence but did not change the resulting sequence of the protein. The mutagenized plasmids were used to transform either the JA221 or XL1-Blue strains of E. coli and recombinant colonies selected for chloramphenicol resistance. Chloramphenicol resistant colonies were further tested for the presence of the desired unique restriction enzyme site by DNA restriction mapping analysis. The resulting IFN-beta plasmid, pCMG275.8, contained the full set of unique restriction enzyme cleavage sites and the DNA sequence of the gene was verified. The full DNA sequence (SEQ ID NO: 1) of the modified, his-tagged interferon beta gene, together with the protein coding sequence (SEQ IDNO: 2) , are given in Figure 10.

The full set of alanine substitution mutations are depicted in Table 1 (below). The names of the mutants specify the structural regions (helices and loops) in which the mutations were introduced. The entire panel of alanine (serine) substitutions results in mutation of 65 of the 165 amino acids of human IFN-beta.

The panel of mutants was created from pCMG275.8 by replacing segments of DNA

TABLE 1

Positions of alanine substitution mutations of ^{HU}IFN-β

```
          1         10        20        30        40        50
          |  ...  .  |  .  .. .|  ..  ..|. ..  .. .|  .  .....|...
IFN-β MSYNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKE
A1    -A-AA--A--A-------------------------------------------
A2    --------------AA-AA--AA-------------------------------
AB1   ----------------------------AAA-AA---------------------
AB2   ------------------------------------AA-A--A------------
AB3   ---------------------------------------------AAAAA-AAA
      |--------helix A---------||----------AB loop-----------|

                60        70        80        90        100
                |  .  .. |  ...    |  ..  ..  .|  .  .  .. .. ...
IFN-β  DAALTIYEMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKE
B1     ----------A--AS-------------------------------------------
B2     ----------------AAA--------------------------------------
C1     --------------------------AS--AA--S----------------------
C2     ------------------------------------A---A--AA-----------
CD1    ---------------------------------------------AA--AAA
       |---------helix B--||-------------------------||CD loop--

          110       120       130       140       150       160
          |..  .  . .|       .|..  . .. .|.      .|  .  .  .|
IFN-β  DFTRGALMSSLHLKRYYGRILHYLKAKEYSHCAWTIVRVEILRNFYRINRLTGYLRN
CD2    AA-A--A--A-------------------------------------------------
D      ------------------A-AA--A---------------------------------
DE1    --------------------------AA-------------------------------
DE2    ----------------------------AA-----------------------------
E      ------------------------------------A---A--A--A-----------
       CD loop-| |-----helix D----|       |--------helix E----------|
```

The line designated IFN-β shows the wild type human IFN-β sequence. Alanine or serine substitutions of the IFN-β residues are shown for each of the mutants and dashes, below relevant regions, indicate wild type sequences. The helices and loop structures are indicated as solid lines below the mutants. The DE loop spans the gap between the D and E helices. Two additional alanine substitution mutants (H93A, H97A and H121A) were generated and analyzed in antiviral assays to assess the effects of mutating these histidines, which chelate zinc in the crustal structure dimer. Both of these mutants retained full wild type activity in antiviral assays, suggesting that zinc-mediated dimer formation is not important for IFN-β activity.

CD2	SEQ ID NO:19 BET-096	CTAGAAGAAAAACTGGAGAAAGAAGCAGCTACCGCTGGAAAAGCAATGA GCGCGCTGCACCTGAAAAGA
	SEQ ID NO:20 BET-106	TATTATGGGAGGATTCTGCATTACCTGAAGGCCAAGGAGTACTCACACTGT
D1	SEQ ID NO:21 BET-108	CATGAGCAGTCTGCACCTGAAAAGATATTATGGGGCAATTGCTGCATACCTG GCAGCCAAGGAGTACTCACACTGT
DE1	SEQ ID NO: 22 BET-116	CATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCATTACCTG AAGGCCGCTGCATACTCACACTGTGCCTGGACGAT
DE2	SEQ ID NO: 23 BET-118	CATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCATTACCTGA AGGCAAAGGAGTACGCTGCATGTGCCTGGACGAT
E1	SEQ ID NO:24 BET-104	CGTCAGAGCTGAAATCCTAGCAAACTTTGCATTCATTGCAAGACTTACAG

B. Construction of EBNA 293 expression plasmids

The wild type and mutant IFN-beta genes, fused to the VCAM-1 signal sequence, his tag and enterokinase cleavage site, were gel purified as 761 base pair NotI and BamHI restriction fragments. The purified genes were subcloned into NotI and BamHI cleaved plasmid vector pDSW247, as depicted in the schematic. Plasmid pDSW247 is an expression vector for transient expression of protein in human EBNA 293 kidney cells (Invitrogen, Carlsbad, CA). It contains the cytomegalovirus early gene promoter and EBV regulatory elements which are required for high level gene expression in that system, as well as selectable markers for E. coli (ampicillin resistance) and EBNA 293 cells (hygromycin resistance) as seen in the cloning strategy schematic (below). The ligated plasmids were used to transform either JA221 or XL1-Blue E. coli cells and ampicillin resistant colonies were picked and tested for inserts by restriction map analysis. Maxiprep DNA was made and the sequence of the inserts was verified by DNA sequencing. Positive clones displaying the desired mutagenized sequences were used to transfect human EBNA 293 kidney cells as described below.

purified IgG, antibodies had been raised to purified human IFN-beta-1a) to coat 96-well ELISA plates and a biotinylated form of the same polyclonal rabbit IgG was used as a secondary reagent to allow interferon detection using streptavidin-linked horseradish peroxidase (HRP: Jackson ImmunoResearch, W. Grove, PA). A dilution series of interferon-beta-1a was used to generate standard concentration curves. The his-IFN-beta containing conditioned media from the EBNA transfectants were diluted to obtain samples with concentrations ranging between 10ng/ml and 0.3ng/ml in the ELISA assay. To confirm the concentrations of the IFN-beta in media determined by ELISA, western blot analysis was performed. Reduced culture supernatants and IFN-beta-1a standards were subjected to SDS-PAGE on 10-20% gradient gels (Novex, San Diego, CA) and blotted onto PDVF membranes. Immunoreactive bands were detected with a rabbit polyclonal anti-IFN-beta-1a antiserum (#447, Biogen, Inc., a second antiserum that had been raised against IFN-beta-1a), followed by treatment with HRP-linked donkey anti-rabbit IgG (Jackson ImmunoResearch).

D. Assessing the Interferon-beta Mutants for Receptor Binding

The receptor binding properties of the Interferon-beta mutants described in C were assessed using two different binding assays. One assay measured binding of the interferon-beta mutants to a fusion protein, IFNAR2/Ig, comprising the extracellular domain of the human IFNAR2 receptor chain fused to part of the constant region of a human IgG. IFNAR2-Fc was expressed in chinese hamster ovary (CHO) cells and purified by protein A sepharose affinity chromatography according to the instructions of the manufacturer (Pierce Chem. Co., Rockford, IL, catalog #20334). The binding of interferon-beta mutants to IFNAR2-Fc was measured in an ELISA format assay. ELISA plates were prepared by coating flat-bottomed 96 well plates overnight at 4° C with 50 µl/well of mouse anti-human IgG1 monoclonal antibody (CDG5-AA9, Biogen, Inc.) at 10 µg/ml in coating buffer (50mM $NaHCO_3$, 0.2mM $MgCl_2$, 0.2mM $CaCl_2$, pH 9.6). Plates were washed twice with PBS containing 0.05% Tween-20, and blocked with 0.5% non-fat dry milk in PBS for 1 hour at room temperature. After two more washes, 50 µl of 1 µg/ml IFNAR2-Fc in 0.5% milk in PBS containing 0.05% Tween-20 was added to each well and incubated for 1 hour

resuspended in 300 µl FACS buffer containing 0.5% paraformaldehyde, and transferred into 12x75mm polystyrene tubes (Falcon 2052). The samples were then analyzed by flow cytometry on a FACScan (Becton Dickinson). Data were plotted as mean channel fluorescence intensity (MFCI) versus the concentration of interferon-beta mutant; binding affinities were defined as the concentration of interferon-beta mutant giving 50% inhibition of antibody staining. Each mutant was tested multiple times. Figure 2 shows the receptor binding affinities for each interferon-beta mutant, determined by this method, expressed as a percentage of the affinity measured for His_6-wild-type interferon-beta-1a in each experiment.

E. Assessing the Interferon-beta Mutants for Function

The interferon-beta mutants were also tested for functional activity using *in vitro* assays for antiviral activity and for the ability of the interferon-beta to inhibit cell proliferation. A minimum of three antiviral assays, each with triplicate data points, were performed on each mutant. His_6-wild-type interferon-beta-1a was included as a reference in every experiment. The antiviral assays were performed by treating A549 human lung carcinoma cells (ATCC CCL 185) overnight with 2-fold serial dilutions of mutant interferon-beta at concentrations that spanned the range between full antiviral protection and no protection from viral cell killing. The following day, the cells were challenged for two days with encephalomyocarditis virus (ECMV) at a dilution that resulted in complete cell killing in the absence of interferon. Plates were then developed with the metabolic dye MTT (2,3-bis[2-Methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxyanilide) (M-5655, Sigma, St. Louis, MO). A stock solution of MTT was prepared at 5 mg/ml in PBS and sterile filtered, and 50 µl of this solution was diluted into cell cultures (100 µl per well). Following incubation at room temperature for 30 - 60 minutes, the MTT/media solution was discarded, cells were washed with 100 µl PBS, and finally the metabolized dye was solubilized in 100 µl 1.2N hydrochloric acid in 90% isopropanol. Viable cells (as evidenced by the presence of the dye) were quantified by absorbance at 450 nm. Data were analyzed by plotting absorbance against the concentration interferon-beta mutant, and the activity of each mutant was defined as the concentration at which 50% of the cells were

assume that variations in the activities of mutants A1-E, compared to his tagged-wild-type interferon-beta-1a, are qualitatively and quantitatively about the same as the effects that these same mutations would have in the absence of the N-terminal his tag. The equivalent assumption for tagged or fusion constructs of other soluble cytokines is commonly held to be true by practitioners of the technique of alanine scanning mutagenesis, especially when the in vitro functional activity of the tagged or fusion construct is close to that of the wild-type cytokine as is the case here. See, for example, Pearce K.H. Jr, et al., *J. Biol. Chem.* 272:20595-20602 (1997) and Jones J.T., et al., *J. Biol. Chem.* 273:11667-11674 (1998)

The data shown in Figures 1-4 suggests three types of effects that were caused by the targeted mutagenesis. These effects may be advantageous for interferon drug development under certain circumstances. The three types of effect are as follows: (a) mutants with higher antiviral activity than that of wild-type interferon-beta-1a (e.g. mutant C1); (b) mutants which display activity in both antiviral and antiproliferation assays, but for which antiproliferation activity is disproportionately low with respect to antiviral activity, compared to wild-type interferon-beta-1a (e.g., mutants C1, D and DE1); and (c) functional antagonists (e.g., A1, B2, CD2 and DE1), which show antiviral and antiproliferative activities that are disproportionately low with respect to receptor binding, compared to wild-type interferon-beta-1a. It can be seen that some mutants fall into more than one class. These classes are reviewed below. While we have characterized these classes of mutants with respect to those examples listed, it should be appreciated that other mutations in these regions may result in similar, or even enhanced effects on activity:

a) Mutant C1 possesses antiviral activity that is approximately 6-fold greater than that of wild-type his tagged-interferon-beta-1a. This mutant and others of this type are predicted to be useful in reducing the amount of interferon-beta that must be administered to achieve a given level of antiviral effect. Lowering the amount of administered protein is expected to reduce the immunogenicity of the protein and may also reduce side-effects from non-mechanism-based toxicities. Mutations in this class are predicted to be advantageous in situations where the therapeutic benefit of interferon-beta administration results from its antiviral effects, and where antiproliferative effects contribute to toxicity or to unwanted side-effects.

(c) Mutants with antiviral and antiproliferative activities that are low with respect to receptor binding, as compared to wild-type his tagged-interferon-beta-1a (see Table 4 below). Mutant A1 displays antiviral and antiproliferative activities that are 2.0-fold and 1.8-fold higher than that observed for wild-type his tagged-interferon-beta-1a, but binds to the cognate receptor on Daudi cells with an affinity that is 29-fold higher than wild-type. The binding of this mutant to the IFN-beta receptor is thus enhanced approximately 15-fold compared to the antiviral and antproliferation activities of the protein. Similarly, mutants B2, CD2 and DE1 show enhancements of binding over antiviral activity of 4.6-, 4.6- and 18-fold, respectively, and over antiproliferation activity of 3.5-, 15- and 54-fold. These proteins are predicted to be useful as functional antagonists of the activity of endogenous IFN-beta, and possibly of other endogenous Type I interferons, because they have the ability to bind to and occupy the receptor, and yet generate only a small fraction of the functional response in the target cells that would be seen with wild type IFN-beta.

TABLE 4.

Mutant	Antiviral Activity (AV) (% wt)	Antiproliferative Activity (AP) (% wt)	Cell Binding Activity (% wt)	Binding/AV	Binding /AP
A1	200	180	2900	15	16
B2	7.1	9.2	33	4.6	3.5
CD2	150	46	690	4.6	15
DE1	26	8.5	460	18	54

G. Mutein Relationship to Three Dimensional Structure of Interferon

While published crystal structures for a non-glycosylated form of murine interferon beta (T. Senda, S. Saitoh and Y. Mitsui. Refined Crystal Structure of Recombinant Murine Interferon-β at 2.15 Å Resolution. *J. Mol. Biol.* **253**: 187-207 (1995)) and for human interferon alpha-2b (R. Radhakrishnan, L.J. Walter, A. Hruza, P. Reichert, P.P Trotta, T.L. Nagabhushan and M.R. Walter. Zinc Mediated Dimer of Human Interferon-α2b Revealed by X-ray Crystallography. *Structure.* **4**: 1453-1463 (1996)) had provided models for the

5.5, 75 mM NaCl, and the product was eluted with 30 mM sodium phosphate pH 6.0, 600 mM NaCl. Elution fractions were analyzed for their absorbance values at 280 nm and the concentration of interferon in the samples estimated from the absorbance using an extinction coefficient of 1.51 for a 1 mg/ml solution.

To a 1 mg/ml solution of the interferon-beta-1a from the SP eluate, 0.5 M sodium phosphate pH 6.0 was added to 50 mM, sodium cyanoborohydride (Aldrich, Milwaukee, WI) was added to 5 mM, and 20K PEG aldehyde (Shearwater Polymers, Huntsville, AL) was added to 5 mg/ml. The sample was incubated at room temperature for 20 hours. The pegylated interferon was purified from reaction products by sequential chromatography steps on a Superose ® 6 FPLC sizing column (Pharmacia) with 5 mM sodium phosphate pH 5.5, 150 mM NaCl as the mobile phase and SP-Sepharose ® FF. The sizing column resulted in base line separation of modified and unmodified interferon beta (chromatograph not presented here). The PEG-interferon beta-containing elution pool from gel filtration was diluted 1:1 with water and loaded at 2 mg interferon beta /ml resin onto an SP-Sepharose ® column. The column was washed with 5 mM sodium phosphate pH 5.5, 75 mM NaCl and then the pegylated interferon beta was eluted from the column with 5 mM sodium phosphate pH 5.5, 800 mM NaCl. Elution fractions were analyzed for protein content by absorbance at 280 nm. The pegylated interferon concentration is reported in interferon equivalents as the PEG moiety did not contribute to absorbance at 280 nm.

B. Biochemical Characterization of PEGylated Interferon.

Samples were analyzed for extent of modification by SDS-PAGE (gel not presented here). Addition of a single PEG resulted in a shift in the apparent mass of interferon from 20 kDa to 55 kDa which was readily apparent upon analysis. In the pegylated sample there was no evidence of unmodified interferon-beta-1a nor of higher mass forms resulting from the presence of additional PEG groups. The presence of a single PEG was verified by MALDI mass spectrometry. The specificity of the pegylation reaction was evaluated by peptide mapping. 20 µg aliquots of pegylated, and unmodified interferon-beta-1a as a control, in 240 µL of 200 mM Tris HCl pH 9.0, 1 mM EDTA were digested with 1.5 µg of lysyl endoproteinase from Achromobacter (Wako Bioproducts, Richmond, VA) for 3-4 hours at 27 ° C. 200 mg of guanidine HCl was added to each sample and the cleavage products

of interferon-beta-1a is about 10 times greater than the specific activity of interferon-beta-1b and therefore PEGylated interferon-beta-1a is significantly more active than any interferon-beta-1b product.

Interferon-beta-1a was also PEGylated with a 5K PEG-aldehyde moiety that was purchased from Fluka, Inc. (Cat. No. 75936, Ronkonkoma, NY) following the same protocol described for modification with 20K PEG aldehyde except that the reaction contained 2 mg/ml of the 5K PEG. Modification with the 5K PEG was also highly specific for the N-terminus and did not alter the antiviral activity of interferon-beta-1a. Like the 20K adduct, the 5K PEG inteferon-beta-1a was indistinguishable from the unmodified interferon-beta-1a in the antiviral assay.

EXAMPLE 3: **PEGylation Protects Interferon-beta-1a from Stress-Induced Aggregation**

Aggregation of interferon beta has a deleterious effect on activity. Previously, we have shown that glycosylation has a dramatic effect on stability of interferon-beta-1a versus nonglycosylated forms of interferon beta and inferred that glycosylation contributes to the higher specific activity of interferon-beta-1a (Runkel L. et al, Pharm. Res. 15: 641-649). To investigate whether conjugation with a polyalkylene glycol polymer might further stabilize interferon beta, we subjected the PEGylated interferon-beta-1a to thermal stress using the following protocol:

Thermal denaturation was carried out using a CARY 3 UV-visible spectrophotometer fitted with a computer controlled, thermolectrically heated cuvette holder. Solutions of interferon-beta-1a in 20 mM HEPES pH7.5, 20mM NaCl were equilibrated at 25 ° C in a 1 ml cuvette. The temperature of the cuvette holder was then ramped from 25 ° C to 80 ° C at a rate of 2 ° C/min, and the denaturation of the protein followed by continuous monitoring of absorbance at 280 nm. The mid-point of the cooperative unfolding event, Tm , was obtained from the melting curves by determining the temperature at which the measured absorbance was mid-way between the values defined by lines extrapolated from the linear regions on each side of the cooperative unfolding transitions.

Results from this analysis are shown in Figure 8. Whereas the non-PEGylated-interferon-beta-1a denatured and aggregated with a 50% point of transition at 60 ° C, there was

levels of activity in serum and those remaining after 48 h, we infer that the half life of the PEGylated interferon is extended when compared to the half life of unmodified interferon-beta-1a. A second highly significant finding from the study was that very little of the PEGylated form was lost during the distribution phase, as evidenced by the similar high levels of activity at time 0 and after 60 min. The data indicate that, unlike the control interferon-beta-1a, the distribution of the PEGylated product is largely limited to the vasculature.

EXAMPLE 5: **Comparative Pharmacokinetics and Pharmacodynamics in Primates (General Protocols)**

Comparative studies are conducted with polymer-interferon-beta 1a conjugates and native interferon-beta 1a (as non formulated bulk intermediate interferon-beta-1a in sodium phosphate, and NaCl, pH 7.2) to determine their relative stability and activity in primates. In these studies, the pharmacokinetics and pharmacodynamics of the polymer-interferon-beta 1a conjugate in primates is compared to that of native interferon-beta 1a and reasonable inferences can be extended to humans.

Animals and Methods

Study Design

This is a parallel group, repeat dose study to evaluate the comparative pharmacokinetics and pharmacodynamics of conjugated and unconjugated interferon-beta-1a.

Healthy primates (preferably rhesus monkeys) are used for this study. Prior to dosing, all animals will be evaluated for signs of ill health by a Lab Animal Veterinary on two occasions within 14 days prior to test article administration; one evaluation must be within 24 hours prior to the first test article administration. Only healthy animals will receive the test article. Evaluations will include a general physical examination and pre-dose blood draws for baseline clinical pathology and baseline antibody level to interferon-beta-1a. All animals will be weighed and body temperatures will be recorded within 24 hours prior to test article administrations.

control wells containing neither interferon beta of any kind nor EMC; and virus control wells contain cells and EMC but no interferon beta. Control plates containing the standard and samples are also prepared to determine the effect, if any, of the samples on cell growth. These plates are stained without the addition of virus.

Samples and standards are tested in duplicate on each of two replicate assay plates, yielding four data points per sample. The geometric mean concentration of the four replicates is reported. The limit of detection in this assay is 10 units (U)/ml.

Serum concentrations of neopterin are determined at the clinical pharmacology unit using commercially available assays.

Pharmacokinetic and Statistical Methods

RstripTM software (MicroMath, Inc., Salt Lake City, UT) is used to fit data to pharmacokinetic models. Geometric mean concentrations are plotted by time for each group. Since assay results are expressed in dilutions, geometric means are considered more appropriate than arithmetic means. Serum interferon levels are adjusted for baseline values and non-detectable serum concentrations are set to 5 U/ml, which represents one-half the lower limit of detection.

For IV infusion data, a two compartment IV infusion model is fit to the detectable serum concentrations for each subject, and the SC data are fit to a two compartment injection model.

The following pharmacokinetic parameters are calculated:

(i) observed peak concentration, C_{max} (U/ml);

(ii) area under the curve from 0 to 48 hours, AUC using the trapezoidal rule;

(iii) elimination half-life;

and, from IV infusion data (if IV is employed):

(iv) distribution half-life (h);

(v) clearance (ml/h)

(vi) apparent volume of distribution, Vd (L).

WinNonlin (Scientific Consulting Inc., Apex, NC) software is used to calculate the elimination half-lives after SC and IM injection.

analysis data. Neither the integrity nor interpretation of the data was affected by these differences.

Results and Discussion

Within each route of administration, pegylated IFN beta-1a exhibited higher bioavailability (as measured by the area under the serum concentration-time curve). In addition the pegylated IFN beta-1a had a higher absolute bioavailability as compared to IFN beta-1a when administered by the SC route. We summarize the pharmacokinetic parameters in Table 5. Administration of pegylated IFN beta-1a by both IV and SC routes results in an increase in the half-life as well as the AUC of IFN beta-1a.

TABLE 5:

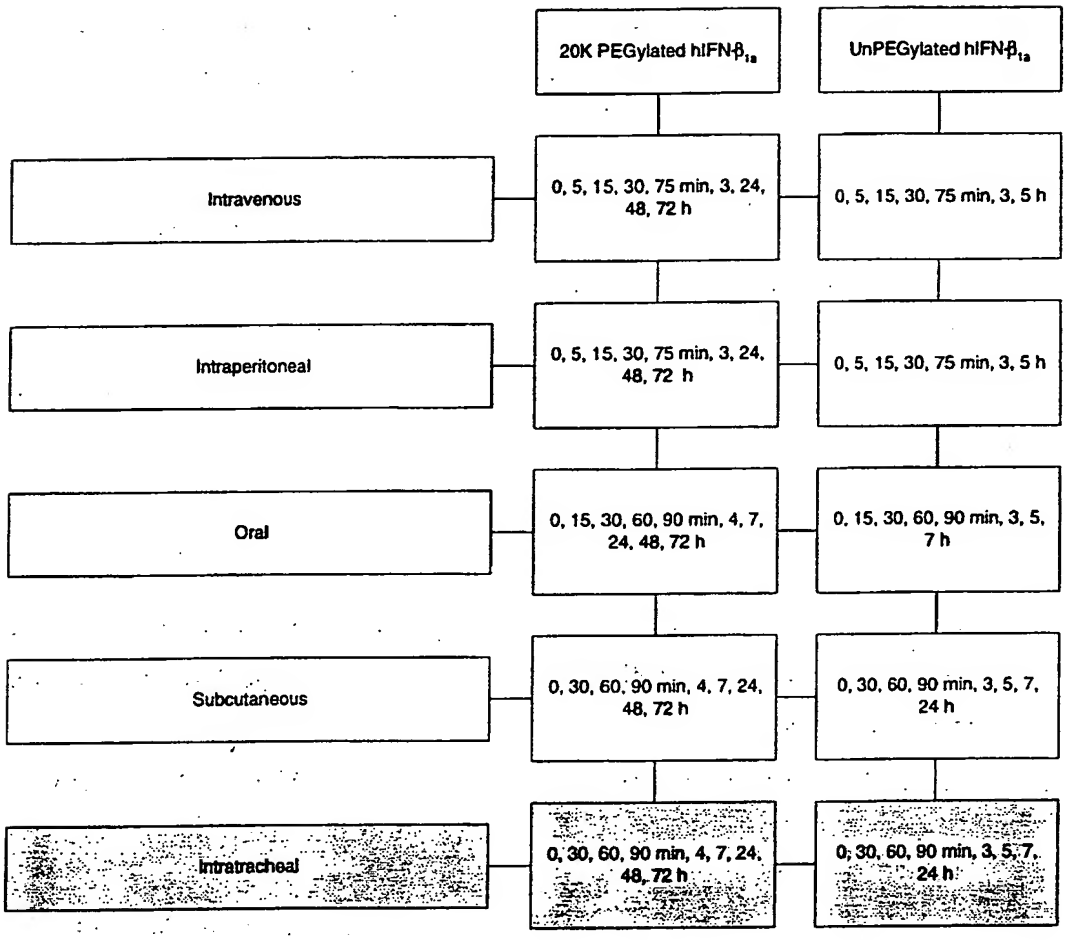
Mean (± Std. Dev.) BG9418 Pharmacokinetic Parameters Following IV or SC (Dose 1) Administration of 1 MU/kg of IFN b-1a or Pegylated IFN B-1a to Rhesus Monkeys[a]

Formulation (Route of Administration)	C_{max}	T_{max}	AUC U*hr/mL	CL (mL/kg)	Vss (mL/kg)	$T_{1/2}$
IFN B-1a (IV)	6400 (±0)	0.083 (±0)	4453 (±799)	229 (±38)	543 (±147)	3.2 (±1.4)
Pegylated IFN- b-1a (IV)	10800 (±3811)	0.083 (±0)	34373 (±3601)	29 (±3)	250 (±30)	9.5 (±2.1)
IFN B-1a (SC)	277 (±75)	5.3 (±1.2)	4753 (±3170)	N/A	N/A	10.0 (±2.9)
Pegylated IFN B-1a (SC)	1080 (±381)	3.3 (±1.2)	42283 (±5934)	N/A	N/A	22.0 (±3.4)

[a]n=3

Following IV administration of the first dose, the mean (± std. dev.) peak serum concentrations (Cmax) of IFN beta-1a and pegylated IFN beta- 1a were 6400 (± 0) and 10800 (± 3.5) U/mL, respectively. The mean (± std. dev.) AUC values were 4453 (± 799) and 34373 (± 3601) U*hr/mL, respectively. Following the first SC administration, the mean (± std. dev.) Cmax of IFN beta-1a and pegylated IFN beta-1a were 277 (± 75) and

TABLE 6.


20K PEGylated hIFN-β1a
UnPEGylated hIFN-β1a
Intravenous
0, 5, 15, 30, 75 min, 3, 24, 48, 72 h
0, 5, 15, 30, 75 min, 3, 5 h
Intraperitoneal
0, 5, 15, 30, 75 min, 3, 24, 48, 72 h
0, 5, 15, 30, 75 min, 3, 5 h
Oral
0, 15, 30, 60, 90 min, 4, 7, 24, 48, 72 h
0, 15, 30, 60, 90 min, 3, 5, 7 h
Subcutaneous
0, 30, 60, 90 min, 4, 7, 24, 48, 72 h
0, 30, 60, 90 min, 3, 5, 7, 24 h
Intratracheal
0, 30, 60, 90 min, 4, 7, 24, 48, 72 h
0, 30, 60, 90 min, 3, 5, 7, 24 h

as could an antibody to bFGF). The final volume is adjusted to 0.5 ml in the 24 well plate or 0.2 ml in the 96 well plate.

After seventy-two hours, cells are trypsinized for Coulter counting, frozen for CyQuant fluorescense reading, or labeled with [3H] thymidine. The inhibition of endothelial cell proliferation in vitro by conjugated and unconjugated interferon-beta 1a was comparable, indicating that PEGylation had not interfered with the ability of the interferon to function in this setting.

This in vitro assay tests the human interferon-beta molecules of the invention for effects on endothelial cell proliferation which may be indicative of anti-angiogenic effects in vivo. See O'Reilly, M.S., T. Boehm, Y. Shing, N. Fukal, G. Vasios, W. Lane, E. Flynn, J. Birkhead, B. Olsen, and J. Folkman. (1997). Endostatin: An Endogenous Inhibitor of Angiogensis and Tumor Growth. *Cell* 88, 277-285.

EXAMPLE 9: **In Vivo Model to Test Anti-Angiogenic and Neovascularization Effects of Conjugated Interferon-beta-1a**

A variety of models have been developed to test for the anti-angiogenic and anti-neovascularization effects of the molecules described herein. Some of these models have been described in United States Patents 5,733,876 (Mar. 31,1998: "Method of inhibiting angiogenesis) and 5,135,919 (Aug. 4, 1992:" Method and a pharmaceutical composition for the inhibition of angiogenesis "). Other assays include the shell-less chorioallantoic membrane (CAM) assay of S. Taylor and J. Folkman; Nature, 297, 307 (1982) and R.Crum. S.Szabo and J.Folkman; Science. 230. 1375 (1985); the mouse dorsal air sac method antigiogenesis model of Folkman, J. et al.; J.Exp.Med.,133, 275 (1971) and the rat corneal micropocket assay of Gimbrone, M.A. Jr. et al., J. Natl. Cancer Inst. 52, 413(1974) in which corneal vascularization is induced in adult male rats of the Sprague-Dawley strain (Charles River, Japan) by implanting 500 ng of basic FGF (bovine, R & D Systems, Inc.) impregnated in EVA (ethylene-vinyl acetate copolymer) pellets in each cornea.

Other methods for testing PEGylated murine interferon-beta for anti-angiogenic effects in an animal model include (but are not limited to) protocols for screening new potential anticancer agents as described in the original Cancer Chemotherapy Reports, Part

Fragment: Prepare a 2-4 mm fragment of s.c. donor tumor.

Time: Day 13-15.

Site: Implant the fragment s.c. in the axillary region with a puncture in the inguinal region.

Testing Schedule:

Day 0: Implant tumor. Run bacterial cultures. Test positive control compound in every odd-numbred experiment. Prepare materials. Record deaths daily.

Day 1: Check cultures. Discard experiment if contaminated. Randomize animals. Treat as instructed (on day 1 and on following days).

Day 2: Recheck cultures. Discard experiment if contaminated.

Day 5: Weigh Day 2 and day of initial test agent toxicity evaluation.

Day 14: Control early-death day.

Day 48: Control no-take day.

Day 60: End and evaluate experiment. Examine lungs grossly for tumor.

Quality Control:

Schedule the positive control compound (NSC 26271 (Cytoxan at a dose of 100 mg/kg/injection)) in every odd-numbered experiment, the regimen for which is intraperitoneal on Day 1 only. The lower Test/Control limit for the positive control is 140%. The acceptable untreated control median survival time is 19-35.6 days.

Evaluation:

The parameter measured is median survival time Compute mean animal body weights for Day 1 and Day 5, compute Test/Control ratio for all test groups with. The mean animal body weights for staging day and final evaluation day are computed. The Test/Control ratio is computed for all test groups with > 65 % survivors on Day 5. A Test/Control ratio value <86% indicates toxicity. An excessive body weight change difference (test minus control) may also be used in evaluating toxicity.

Criteria for Activity:

An initial Test/Control ratio greater than or equal to 140% is considered necessary to demonstrate moderate activity. A reproducible Test/Control ratio value of greater than or equal to 150% is considered significant activity.

polyalkylene glycol moiety are arranged such that the physiologically active interferon-beta 1a in the physiologically active interferon-beta composition has an enhanced activity relative to physiologically active interferon-beta 1b, when measured by an antiviral assay.

9. The composition of claim 8, wherein the interferon-beta-1a is coupled to the polymer at a site on the interferon-beta-1a that is an N-terminal end.

10. The composition of claim 8, wherein the interferon-beta-1a is coupled to the polymer at a site on the interferon-beta-1a that is at or near the C-terminal end.

11. The composition of claim 8, wherein the interferon-beta-1a is coupled to the polymer at a site by way of a glycan moiety of the interferon-beta-1a.

12. The composition of claim 8, wherein the interferon-beta-1a is an interferon-beta-1a fusion protein.

13. The composition of claim 12, wherein the interferon-beta-1a fusion protein comprises a portion of an immunoglobulin molecule.

14. The composition of claims 8 or 12, wherein the interferon-beta-1a is a mutant interferon-beta-1a having at least one of the following properties: (a) the mutant has a higher antiviral activity than wild type inteferon beta 1a, wherein the antiviral activity is measured by viral induced lysis of cells; (b) the mutant has, relative to wild type interferon-beta-1a, greater antiviral activity than antiproliferative activity; (c) the mutant binds interferon receptor but has, when compared to wild type interferon-beta-1a, lowered antiviral activity and lowered antiproliferative activity relative to receptor binding activity.

15. A physiologically active interferon-beta composition comprising a physiologically active glycosylated interferon-beta N-terminally coupled to a polymer comprising a polyalkylene glycol moiety, wherein the physiologically active interferon-beta and the polyalkylene glycol moiety are arranged such that the physiologically active interferon-beta in the physiologically active interferon-beta composition has substantially similar activity relative to physiologically active interferon-beta lacking said moiety, when measured by an antiviral assay.

composition comprising said interferon-beta 1a coupled to a polyethylene glycol moiety.

26. The method of claim 25, wherein the interferon-beta-1a is coupled to the polymer at a site on the interferon-beta-1a that is an N-terminal end.

27. The method of claim 25, wherein the interferon-beta-1a is coupled to the polymer at a site on the interferon-beta-1a that is at or near the C-terminal end.

28. The method of claim 25, wherein the interferon-beta-1a is coupled to the polymer at a site by way of a glycan moiety on the interferon-beta-1a.

29. The method of claim 25, wherein the interferon-beta-1a is an interferon-beta-1a fusion protein.

30. The method of claim 29, wherein the interferon-beta-1a fusion protein comprises a portion of an immunoglobulin molecule.

31. The method of claims 25 and 29, wherein the interferon-beta-1a is a mutant interferon-beta-1a having at least one of the following properties: (a) the mutant has a higher antiviral activity than wild type inteferon beta 1a, wherein the antiviral activity is measured by viral induced lysis of cells; (b) the mutant has, relative to wild type interferon-beta-1a, greater antiviral activity than antiproliferative activity; (c) the mutant binds interferon receptor but has, when compared to wild type interferon-beta-1a, lowered antiviral activity and lowered antiproliferative activity relative to its receptor binding activity.

32. A method of prolonging the activity of interferon-beta-1a in an in vivo or in vitro system, comprising coupling said interferon-beta 1a to a non-naturally-occurring polymer moiety to yield a coupled polymer-interferon-beta 1a composition, and introducing the coupled polymer-interferon-beta composition to the in vivo or in vitro system.

33. The method of claim 32, wherein the interferon-beta-1a is coupled to the polymer at a site on the interferon-beta-1a that is an N-terminal end.

34. The method of claim 32, wherein the interferon-beta-1a is coupled to the polymer at a site on the interferon-beta-1a that is at or near C-terminal end.

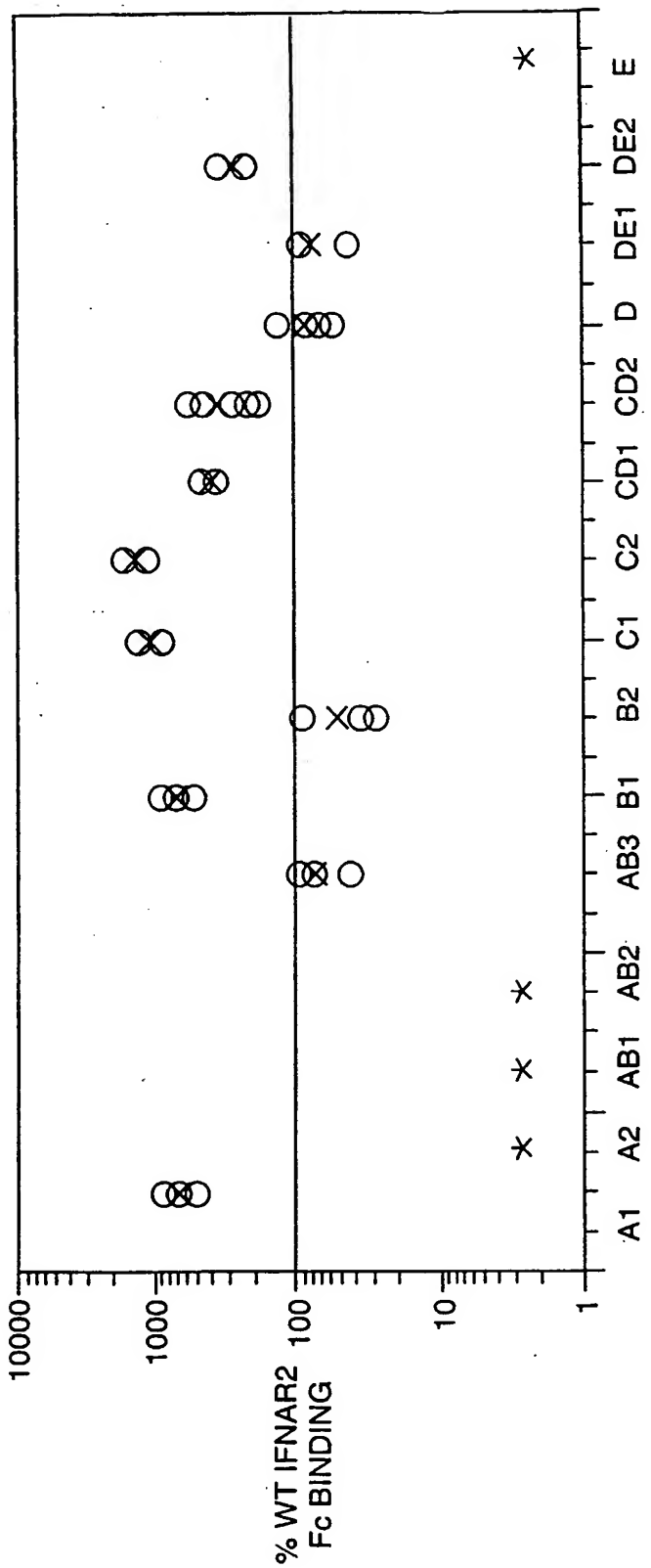

% WT IFNAR2
Fc BINDING
10000
1000
100
10
1
A1
A2
AB1
AB2
AB3
B1
B2
C1
C2
CD1
CD2
D
DE1
DE2
E

FIG. 1

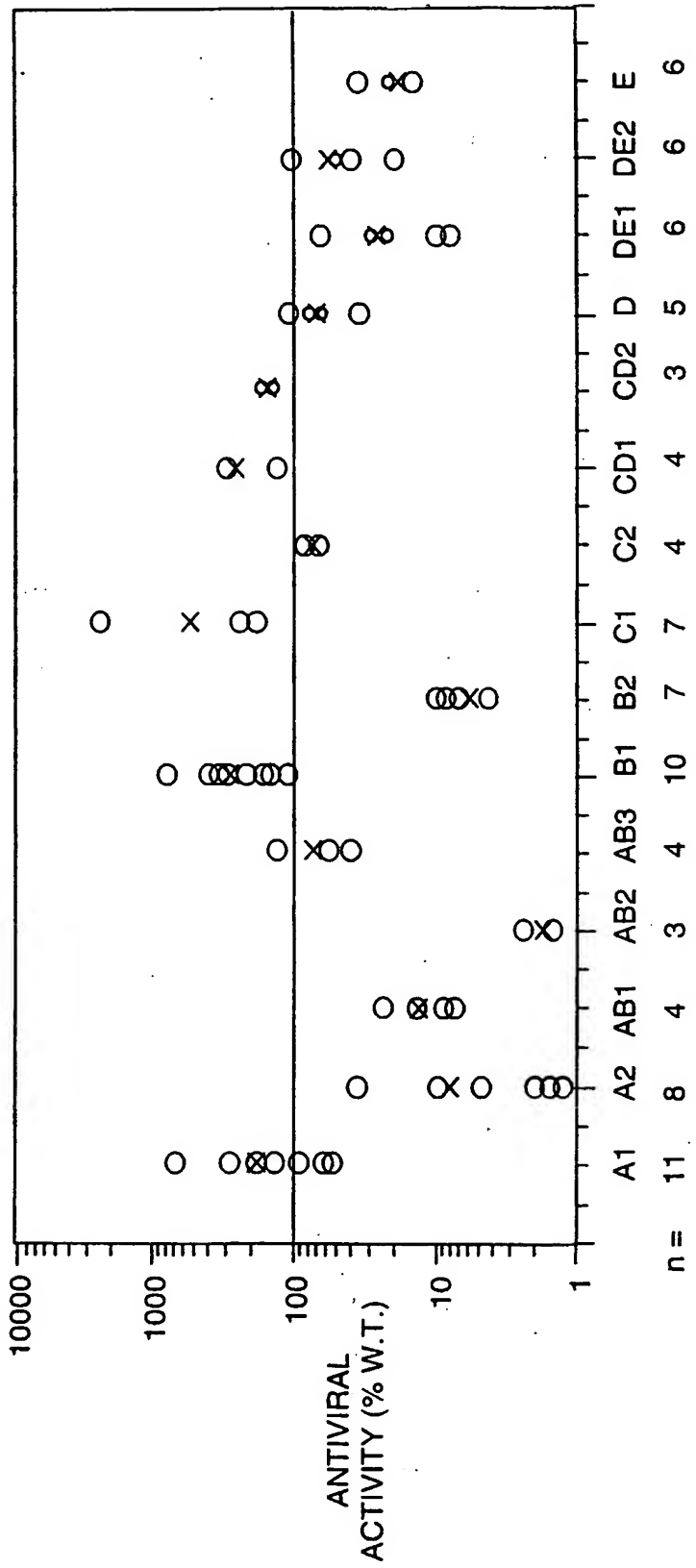

ANTIVIRAL ACTIVITY (% W.T.)
10000
1000
100
10
1
n = 11 8 4 3 4 10 7 4 7 4 3 5 6 6 6
A1 A2 AB1 AB2 AB3 B1 B2 C1 C2 CD1 CD2 D DE1 DE2 E

FIG. 3

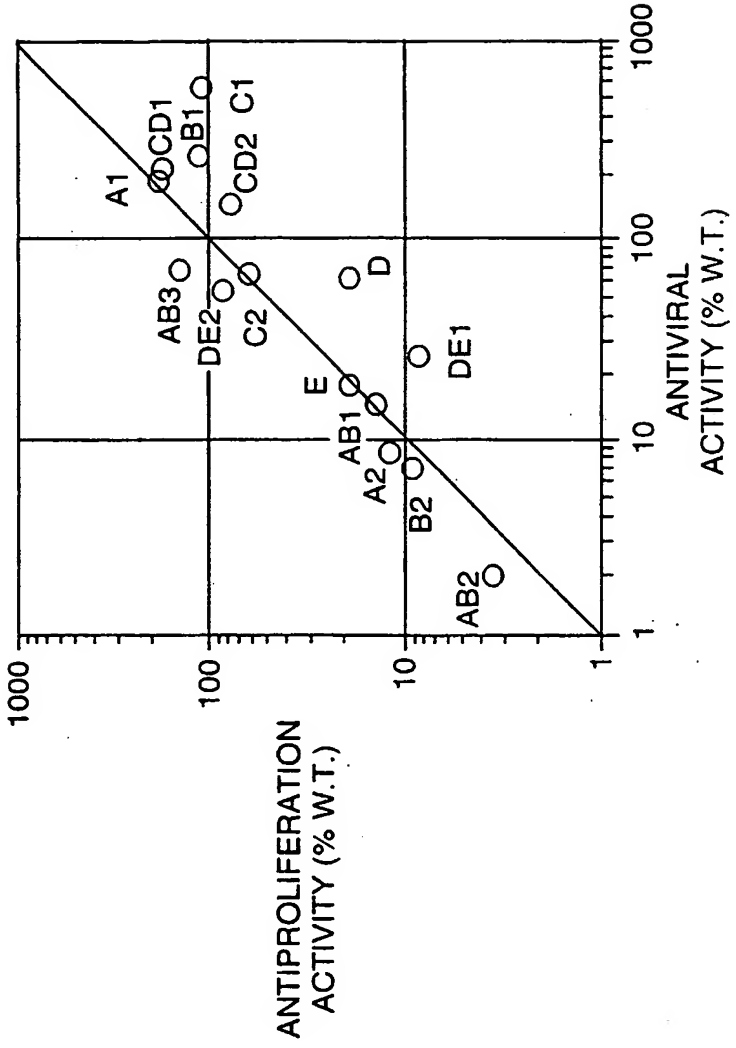

ANTIPROLIFERATION ACTIVITY (% W.T.)
1000
100
10
1
ANTIVIRAL ACTIVITY (% W.T.)
1000
100
10
1
A1
CD1
B1
CD2
C1
AB3
DE2
C2
D
E
DE1
AB1
A2
B2
AB2

FIG. 5

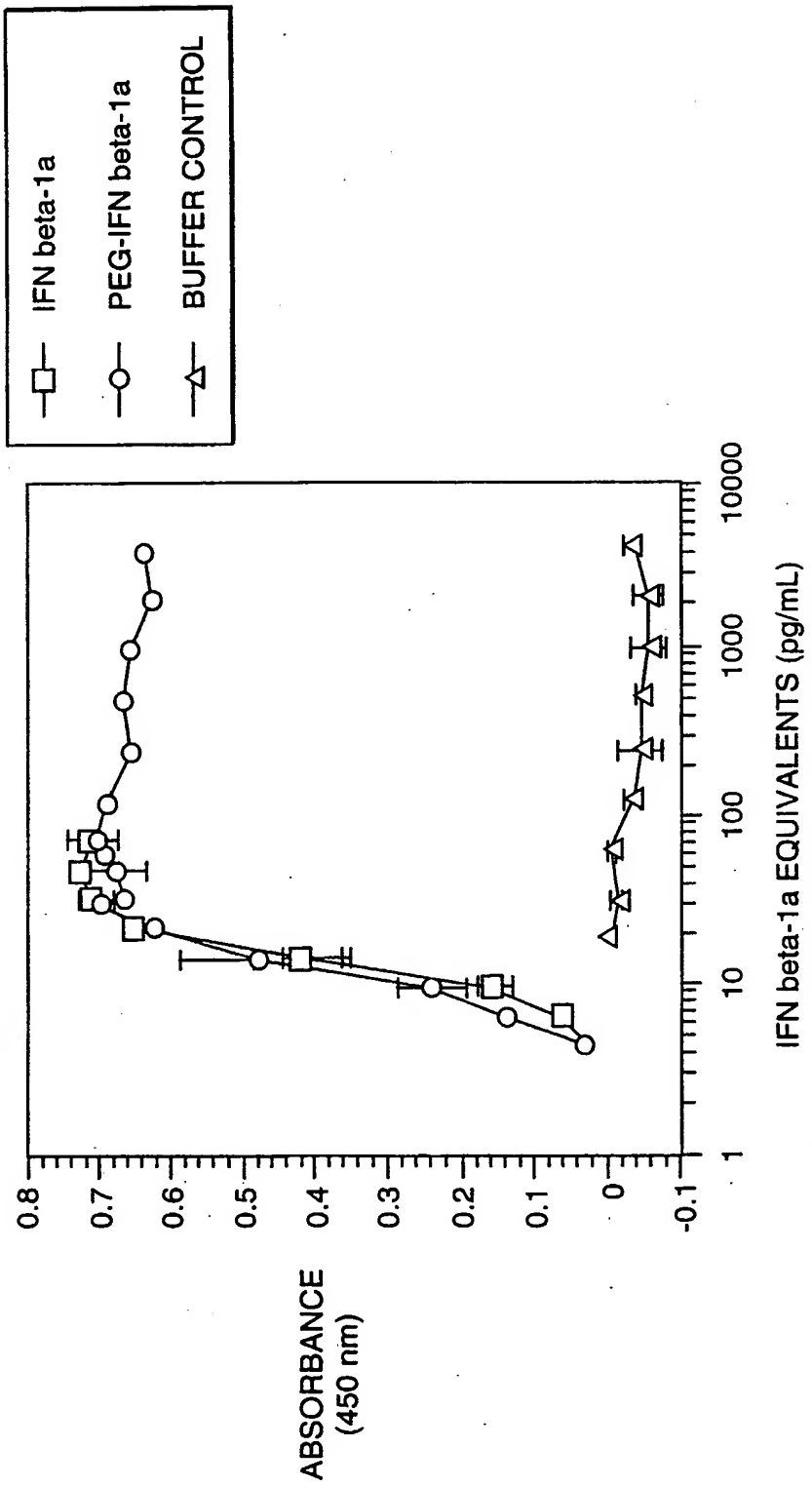

IFN beta-1a
PEG-IFN beta-1a
BUFFER CONTROL
ABSORBANCE (450 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
1
10
100
1000
10000
IFN beta-1a EQUIVALENTS (pg/mL)

FIG. 7

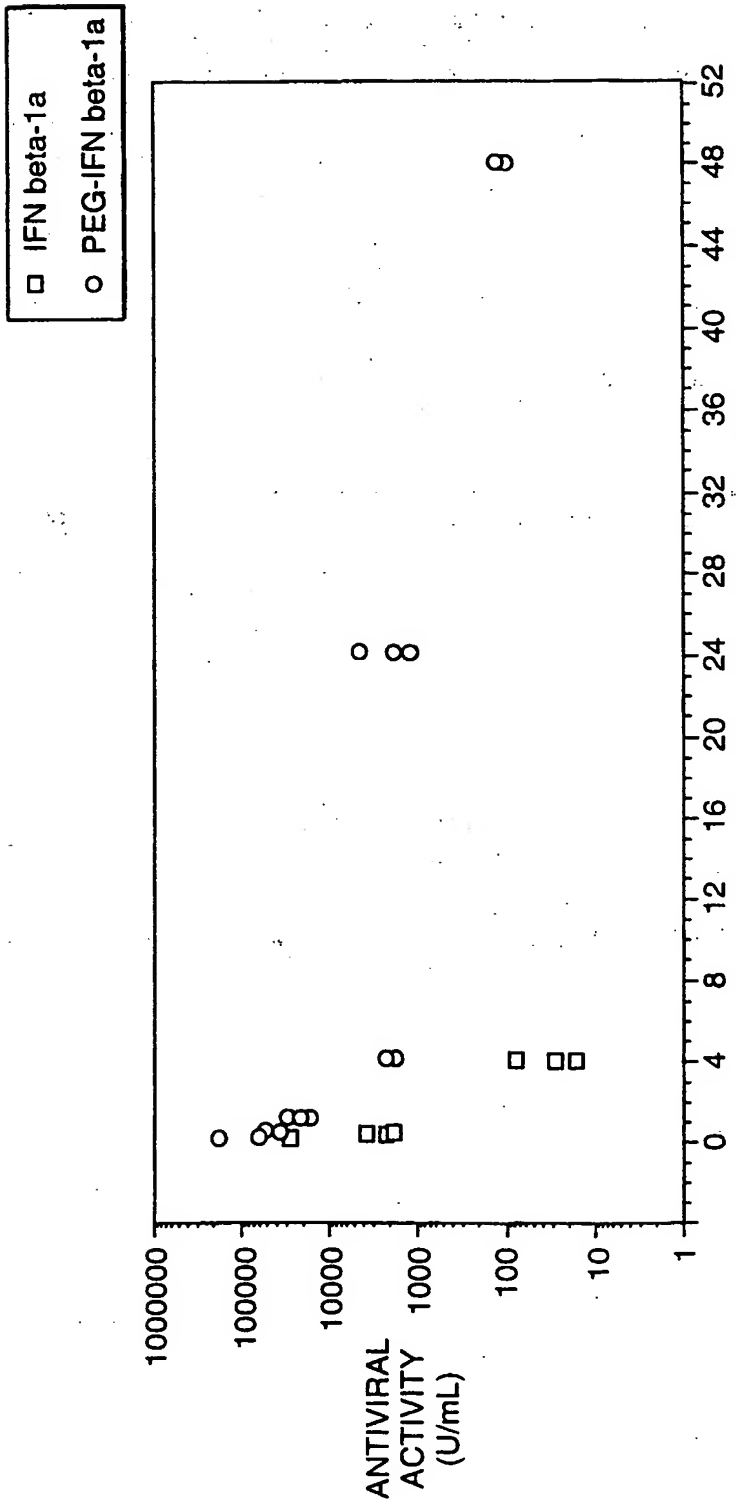

IFN beta-1a
PEG-IFN beta-1a
ANTIVIRAL ACTIVITY (U/mL)
1000000
100000
10000
1000
100
10
1
TIME AFTER INJECTION (HOURS)
0
4
8
12
16
20
24
28
32
36
40
44
48
52

FIG. 9

INTERNATIONAL SEARCH REPORT

Inter. onal Application No
PCT/US 99/24201

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 87 00056 A (CETUS CORP) 15 January 1987 (1987-01-15) abstract page 5, line 28 -page 7, line 9 page 8, line 14 - line 32 page 13, line 15 - line 35 examples VI,VII claims 1-10 --- -/--	1,2,5,6, 9-13, 16-20, 23-30, 32-37, 39,40

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2000	07.07.2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Taylor, G.M.

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/24201

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 25-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 3,4,7,8,14,15,21,22,31,38
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter..onal Application No
PCT/US 99/24201

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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